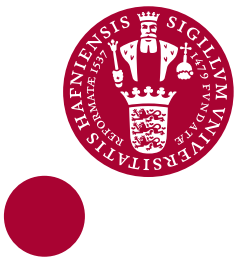

Activation of metabolite receptor GPR91 promotes platelet aggregation and transcellular biosynthesis of leukotriene C4

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BRIEF REPORT

Activation of metabolite receptor GPR91 promotes platelet aggregation and transcellular biosynthesis of leukotriene C_4

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Abstract

Background: Succinate is a Krebs cycle intermediate whose formation is enhanced under metabolic stress, and for which a selective sensor GPR91 has been identified on various cell types including platelets. Platelet-derived eicosanoids play pivotal roles in platelet activation/aggregation, which is key to thrombus formation and progression of atherothrombosis.

Objectives: This study aims to decipher the molecular mechanism(s) and potential involvement of eicosanoids in succinate enhanced platelet activation/aggregation.

Methods: We used liquid chromatography-mass spectrometry (LC-MS)/MS-based lipid mediator profiling to identify eicosanoids regulated by succinate. We ran light transmittance aggregometry and flow cytometry to assess platelet aggregation, P-selectin expression, and platelet-polymorphonuclear leukocyte (PMN) adherence. Various pharmacological tools were used to assess the contributions of GPR91 signalling and eicosanoids in platelet aggregation.

Results: Succinate and two types of synthetic non-metabolite GPR91 agonists—*cis*-epoxysuccinate (cES) and Cmpd131—potentiated platelet aggregation, which was partially blocked by a selective GPR91 antagonist XT1. GPR91 activation increased production of 12-hydroxy-eicosatetraenoic acid (12-HETE), thromboxane (TX) A_2, and 12-hydroxy-heptadecatrienoic acid (12-HHT) in human platelets, associated with phosphorylation of cytosolic phospholipase A_2 ($cPLA_2$), suggesting increased availability of free arachidonic acid. Blocking 12-HETE and TXA_2 synthesis, or antagonism of the TXA_2 receptor, significantly reduced platelet aggregation enhanced by GPR91 signalling. Moreover, platelet-PMN suspensions challenged with succinate exhibited enhanced transcellular biosynthesis of leukotriene C_4 (LTC_4), a powerful proinflammatory vascular spasmogen.

Conclusion: Succinate signals through GPR91 to promote biosynthesis of eicosanoids, which contribute to platelet aggregation/activation and potentially vascular inflammation. Hence, GPR91 may be a suitable target for pharmacological intervention in atherothrombotic conditions.

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KEYWORDS

antiplatelet therapy, eicosanoids, GPR91, platelet aggregation, succinate

1 | INTRODUCTION

GPR91 (Sucnr1), a sensor of extracellular succinate, is expressed in immune, metabolic, and cardiovascular systems.[1] This receptor is closely related to purinergic receptors, in particular $P2Y_1$, and has been strongly implicated in pathogenic processes such as hypertension, ischemic tissue injury, diabetes, and immune responses.[2,3] Platelets play crucial roles in hemostasis, and their activation and aggregation are essential for early clot formation at sites of vascular injury.[4] In addition, platelets participate in immune responses together with leukocytes, and are recognized as critical players in inflammatory processes.[5,6]

Eicosanoids are a series of oxygenated metabolites of arachidonic acid (AA) with potent actions in hemostasis and inflammatory responses.[7,8] Biosynthesis of eicosanoids begins with the release of free AA from membrane phospholipids catalyzed by cytosolic phospholipase A_2 ($cPLA_2$). Further metabolism of AA is mainly directed by two types of oxygenases, cyclooxygenases (COX-1 and COX-2) and lipoxygenases (5-, 12-, and 15-LOX), leading to the generation of prostaglandins, thromboxanes, hydroxy and dihydroxy fatty acids, including the leukotrienes, via enzymes in downstream pathways. Upon activation, platelets rapidly liberate AA by phosphorylated and activated $cPLA_2$ ($p\text{-}cPLA_2$) for eicosanoid synthesis. The major platelet-derived eicosanoids are 12-hydroxy-eicosatetraenoic acid (12-HETE), generated by platelet 12-LOX, and TXA_2 together with equimolar amounts of 12-hydroxy-heptadecatrienoic acid (12-HHT), in a reaction catalyzed by TX synthase.[9,10] Platelets also participate in synthesis of other eicosanoids, especially leukotriene C_4 (LTC_4), via transcellular metabolism of LTA_4 generated along the 5-LOX pathway by neighboring activated leukocytes.[6,11]

Previous work has demonstrated that succinate can potentiate platelet activation.[12,13] Here, we sought to elucidate the molecular mechanism(s) and potential involvement of eicosanoids in this prothrombotic effect using liquid chromatography-mass spectrometry (LC-MS)/MS based lipid mediator profiling, recently identified synthetic GPR91 agonists and antagonists, as well as inhibitors of key enzymes in the eicosanoid cascade. We report a critical role of GPR91 in this response as well as the ability of this metabolite receptor to promote transcellular generation of pro-inflammatory LTC_4.

Essentials

- GPR91 is expressed on platelets and can sense extracellular succinate.
- Succinate enhances platelet aggregation, and release of 12-HETE, TXA_2, and 12-HHT via GPR91 signaling.
- Succinate-GPR91 activation enhances transcellular biosynthesis of LTC4 in platelet PMN suspension.
- GPR91 is a potential target for development of antiplatelet agents.

2 | MATERIALS AND METHODS

2.1 | Reagents

Succinate (sodium succinate dibasic hexahydrate), adenosine 5′-diphosphate (ADP), forskolin, and monoclonal antibody against human β-actin were purchased from Sigma-Aldrich. Polyclonal antibody against GPR91 was from Abcam, while p-ERK, ERK, $p\text{-}cPLA_2$, $cPLA_2$, COX-1 and COX-2 antibodies were from Cell Signaling Technology. Fluorescence-labeled antibodies against P-selectin, CD42a and CD45 were from Becton Dickinson. cES and Cmpd131 were custom synthesized by Enamine, Kiev, Ukraine and compound XT1 by Ramidus AB (Ideon Science Park). ML355, SC-560, acetylsalicylic acid (ASA), SQ29.548, and oxylipin standards were from Cayman Chemical.

2.2 | Blood collection and platelet preparation

For LC-MS/MS analysis, blood was collected from healthy volunteers with BD Vacutainer® ACD-A tubes, while for platelet light transmittance aggregometry (LTA) BD Vacutainer® citrate tubes were used. All volunteers gave informed consent to donate blood (approved by the Ethics Committee of Karolinska Institutet Dnr 94-146) and denied intake of non-steroidal anti-inflammatory drugs for the past 14 days. The whole blood samples were centrifuged at 190 *g* for 10 minutes, and the upper two thirds of platelet rich plasma (PRP) was collected. Further centrifugation at 1400 *g* for 10 minutes yielded platelet poor plasma (PPP).

2.3 | Polymorphonuclear isolation and platelet/PMN co-culture

Human polymorphonuclear (PMN)s were isolated by dextran sedimentation and Ficoll-Paque Premium (GE Healthcare Bio-Sciences) gradient centrifugation, followed by hypotonic lysis of erythrocytes, yielding a purity of >99% PMNs and >96% neutrophils. PMNs were resuspended in phosphate buffered saline (PBS) to 2×10^7 cells/mL and co-incubated with PRP (1:9, *v*/*v*) in the presence of various compounds at 37°C for indicated times. Incubations were stopped with

two volumes of methanol and soluble extracts collected for LC-MS/MS analysis.

2.4 | Eicosanoid extraction and analysis by LC-MS/MS

Samples were diluted 1:1 with extraction buffer (pH = 5.6) and deuterated internal standards were added prior to solid phase extraction (SPE). Compounds were eluted with 2.5 mL of methanol and evaporated under N_2 gas (TurboVap LV; Biotage). Extracts were reconstituted to a final volume of 70 µL in methanol/water (6:1, *v*/*v*) and filtered using membrane spin filters (0.3 µmol/L; Merck Millipore Cooperation). Extracted samples and calibration standards were analyzed on a Acquity UPLC separation module coupled to a Xevo-TQXS triple quadrupole mass spectrometer (Waters Corp.) as previously described.[14]

2.5 | Light transmittance aggregometry

Light transmittance aggregometry analysis was performed on a four-channel aggregometer (PAP-4; Bio-Data Corporation). PRP samples were pre-incubated with vehicle or various inhibitors/antagonists at 37°C. ADP (1 µmol/L) was added with or without GPR91 agonists, and the platelet aggregation was recorded for 8 minutes. The magnitude of final aggregation was analyzed.

2.6 | Flow cytometry

Whole blood samples were incubated with various compounds and fluorescence-labeled antibodies for 20 minutes, fixed with 0.5% paraformaldehyde (PFA) and subjected to flow cytometric analysis of P-selectin surface expression and platelet-PMN aggregates as previously described.[15,16]

2.7 | Western blot

Pelleted platelets were lysed with radioimmunoprecipitation assay (RIPA) buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent protein transfer using an iBlot® 2 system (Thermo Fisher). Protein bands were visualized with enhanced chemiluminescence detection (Thermo Fisher).

2.8 | Statistics

All data are presented as mean ± standard error of mean (SEM). Differences among groups were assessed by one-way, two-way analysis of variance (ANOVA) or Student's *t*-test and a value of $P < .05$ was considered statistically significant.

3 | RESULTS AND DISCUSSION

3.1 | GPR91 signaling enhances ADP-mediated platelet activation and aggregation

Robust expression of GPR91 in platelets was detected by western blot analysis (Figure 1A). We then challenged PRP with ADP together with succinate or any of two synthetic non-metabolite agonists of GPR91, namely cES or Cmpd131. While cES is a conformationally constrained succinate analog, Cmpd131 is a larger backbone modified analog with an amide-linked hydrophobic "side chain," which fits into a side pocket of GPR91.[17,18] Succinate, the endogenous receptor ligand, and the two synthetic agonists all enhanced ADP-induced platelet aggregation (Figure 1). We also synthesized a potent and selective antagonist of GPR91, here denoted XT1, according to a previously published synthetic strategy (Compound 4c in[19]). Pre-incubation with XT1 partially inhibited the effect of succinate lending further support to the notion that GPR91 plays an essential role in this response (Figure 1E).

3.2 | GPR91 agonists elicit 12-HETE, TXA_2, and 12-HHT production from human platelets

In search of lipid mediators that could be involved in succinate-enhanced platelet aggregation, we directly assessed the formation of oxygenated derivatives of polyunsaturated fatty acids (AA, EPA, DHA, and LA) in PRP upon GPR91 activation by LC-MS/MS quantification. Within the panel of analytes, 46 lipid species were detected, with only six AA derivatives (for which the biosynthetic routes are depicted in Figure 2A) influenced by succinate challenge (Figure 2B). By far the most abundant eicosanoids produced by platelets challenged with 1 mmol/L succinate were 12-HETE, TXA_2, and 12-HHT (Figure 2C). Minor amounts of PGD_2, PGE_2, and $PGF_{2\alpha}$ were also detected, probably reflecting COX-1 activity. Similar effects were observed for cES (Figure 2C,D), with a more efficient induction of 12-HETE from platelets by cES than succinate. Succinate and cES increased the phosphorylation of $cPLA_2$ and ERK but not p38 MAPK (Figure 2E), which translates to increased liberation of AA from phospholipids and more substrate for eicosanoid generation.[20] As expected, COX-1 protein level was not influenced by succinate or cES treatments (Figure 2E), and there was no detectable COX-2 protein in any of the treatment groups. Furthermore, succinate potentiated TXA_2 production in ADP-challenged platelets, and pre-incubation with 1 µmol/L XT1, significantly inhibited this effect (Figure 2F). Together, these results indicate that GPR91 ligation primarily leads to activation of ERK and $cPLA_2$, which in

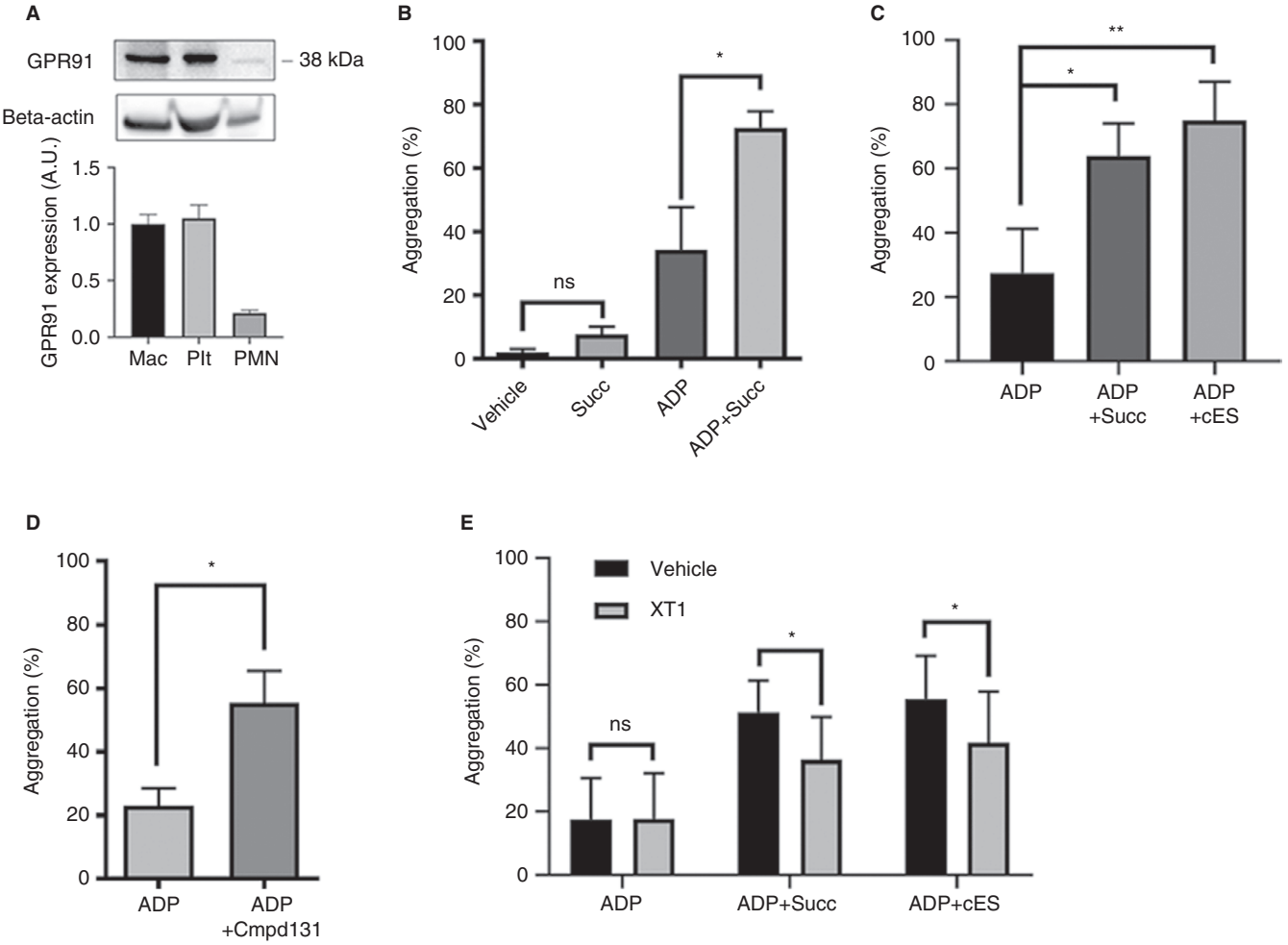

FIGURE 1 GPR91 activation potentiates platelet aggregation. A, Western blot analysis of GPR91 and Beta-actin expression in human monocyte-derived macrophage (Mac), platelet (Plt), and polymorphonuclear (PMN), with band intensity of GPR91 normalized by Beta-actin as indication of GPR91 expression (n = 3). B-D, Vehicle, 1 mmol/L succinate, 1 mmol/L cES, or 100 μmol/L Cmpd131 was added with or without 1 μmol/L adenosine 5′-diphosphate (ADP) and aggregation assessed with the light transmittance aggregometry (LTA) assay (n = 4). E, Platelets were pre-incubated with vehicle or 1 μmol/L XT1 for 1 hour, followed by succinate or cES added with ADP to assess the aggregation magnitude of platelets (n = 3). $^{*}P < .05$, $^{**}P < .01$

turn promotes eicosanoid biosynthesis from human platelets via COX and LOX pathways.

3.3 | Targeting 12-LOX, COX, and TP receptor ameliorate GPR91-potentiated platelet activation

As 12-HETE was one of the major eicosanoids triggered by GPR91 ligation (Figure 2B), a 12-LOX inhibitor-ML355 was used to assess the role of 12-LOX products (12-HpETE and 12-HETE) in succinate-potentiated aggregation induced by ADP. Pre-incubation with ML355 significantly inhibited this succinate potentiation (Figure 3A), suggesting an involvement of 12-LOX. Inhibition of platelet COX-1 by ASA or the selective inhibitor SC-560 also reduced succinate-enhanced platelet aggregation (Figure 3B,C) as did the TP receptor antagonist SQ29.548 (Figure 3D). Moreover, the combination of ML355 with ASA, SC-560, or SQ29.548 showed a more effective inhibitory effect on platelet aggregation than did any of the agents alone. These results suggest that 12-LOX product(s) and TXA_2 are involved in GPR91 potentiated platelet aggregation induced by ADP (Figure 3). Succinate also promoted formation of 12-HHT, a side product of TXA_2 biosynthesis (Figure 2). This compound is the high-affinity ligand of the BLT2 receptor[21] and may elicit responses in neighboring leukocytes. However, it should be noted that the role of BLT2 in cells of the vascular system is poorly understood.

3.4 | Succinate-GPR91 activation enhances platelet-dependent conversion of neutrophil-derived LTA_4 to LTC_4

Platelets not only produce eicosanoids by themselves, but can also cooperate with neighboring cells to produce lipid mediators through transcellular metabolism.[22] Because it has been reported that succinate can increase platelet surface expression of P-selectin, we asked whether this effect would be sufficient to promote transcellular

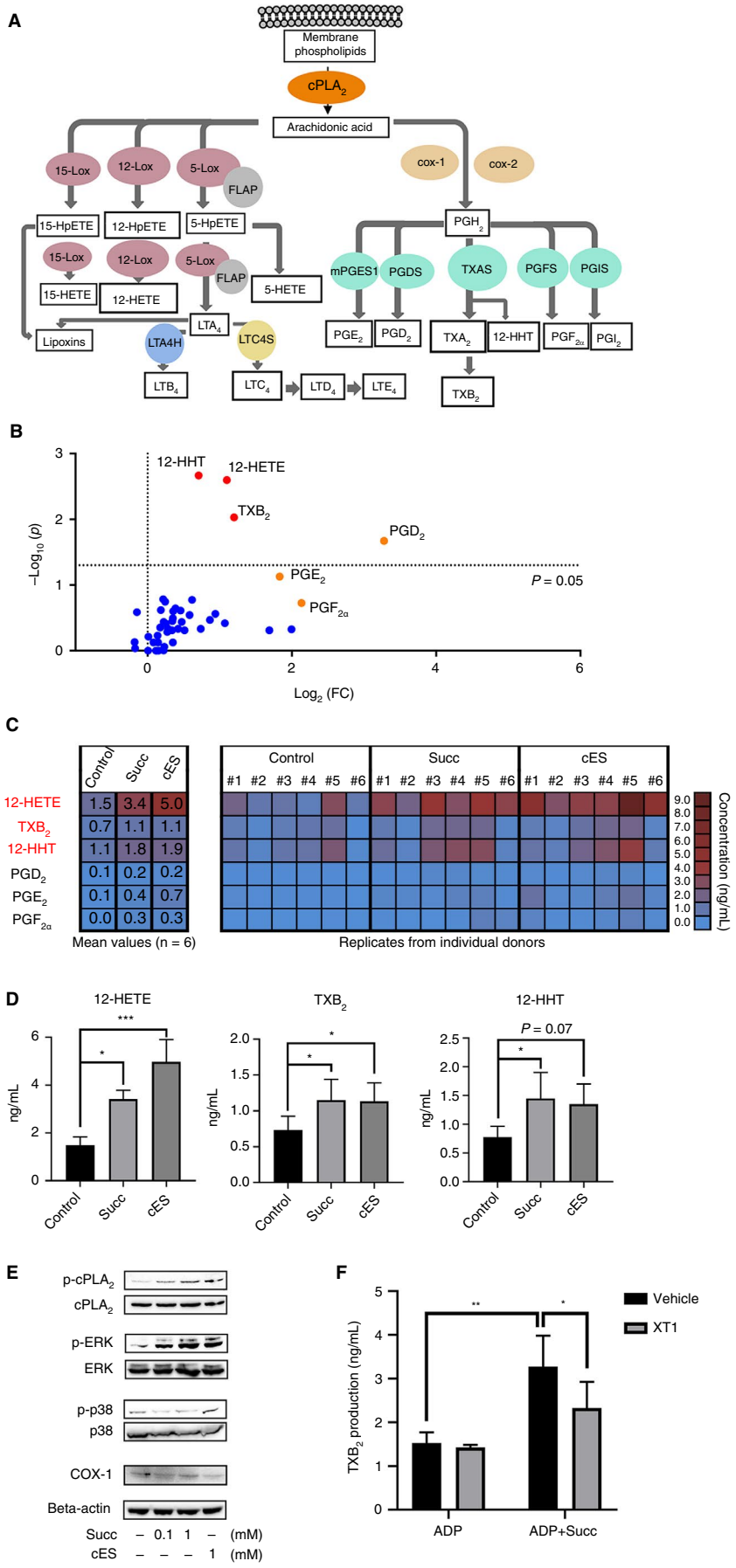

FIGURE 2 Activation of GPR91 triggers 12-hydroxy-eicosatetraenoic acid (12-HETE), thromboxane A_2 (TXA_2), and 12-hydroxy-heptadecatrienoic acid (12-HHT) production from human platelets. A, Biosynthetic routes of eicosanoids from membrane phospholipids. B-D, Platelets were incubated with vehicle, 1 mmol/L succinate, 1 mmol/L cES, or 100 µmol/L Cmpd131 at 37°C for 30 minutes. The reaction was stopped by two volumes of methanol followed by lipid mediator extraction and quantification by liquid chromatography-mass spectrometry (LC-MS)/MS (n = 6). B, Red and orange dots: arachidonic acid (AA) derivatives influenced by succinate challenge. Blue dots: Other oxygenated derivatives of polyunsaturated fatty acids. C, Absolute levels of AA derivatives influenced by succinate or cES (n = 6). D, Comparison of 12-HETE, TXB_2, 12-HHT production from platelets challenged with succinate or cES (n = 6). E, Western blot analysis of total protein from platelets treated with succinate and cES at 37°C for 30 minutes (n = 3). F, Platelets were pre-incubated with vehicle or 1 µmol/L XT1 for 1 hour, followed by adenosine 5′-diphosphate (ADP) added with or without succinate for 30 minutes. TXB_2 level in supernatant was assessed by ELISA (n = 4). *P < .05, **P < .01, ***P < .001

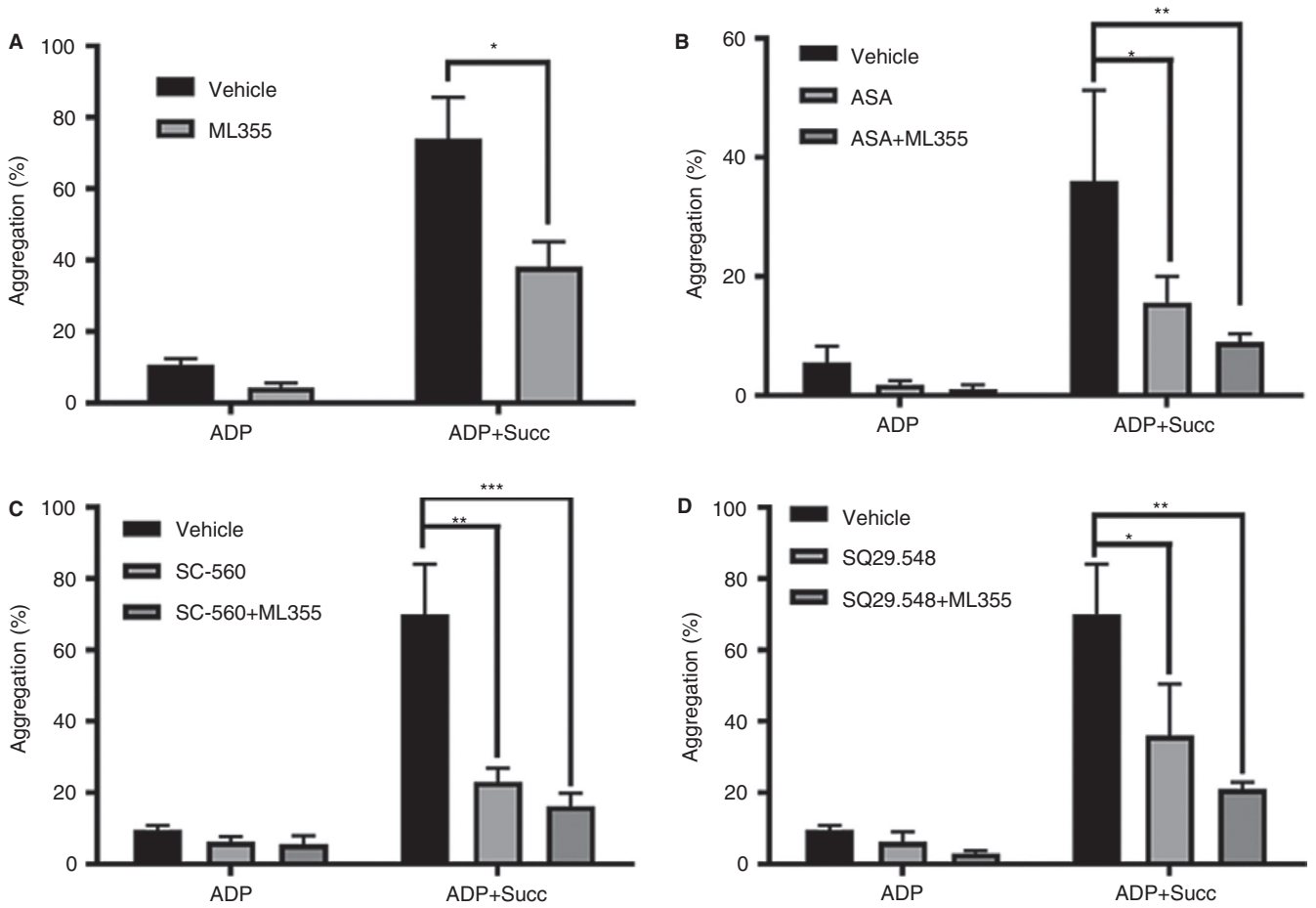

FIGURE 3 TXA_2 and 12-LOX product(s) are involved in succinate-potentiated platelet aggregation. A-D, Platelets were incubated at 37°C for 5 minutes with vehicle or (A) 50 µmol/L ML355 (n = 4), (B) 50 µmol/L ASA with or without 50 µmol/L ML355 (n = 6), (C) 5 µmol/L SC-560 with or without 50 µmol/L ML355 (n = 4), and (D) 5 µmol/L SQ29.548 with or without 50 µmol/L ML355 (n = 4). Afterwards, aggregation was assessed following induction by 1 µmol/L adenosine 5′-diphosphate (ADP) and 1 mmol/L succinate. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$

biosynthetic processes. We chose mixed platelet-PMN suspensions for assessment of transcellular biosynthesis of leukocyte-derived LTA_4 into LTC_4,[11] because there is a distinct partitioning of critical enzymes between platelets (recipient, containing LTC_4 synthase/LTC4S) and PMNs (donor, harboring 5-LOX for production of LTA_4).

In our hands, succinate as well as cES enhanced P-selectin surface expression in human platelets (Figure 4A,B), suggesting an involvement of GPR91 in platelet-neutrophil interactions. Furthermore, platelet PMN suspensions challenged with succinate indeed promoted LTC_4 production as determined by LC-MS/MS analysis (Figure 4C). In contrast, there was no change of LTB_4 or 5-HETE production in succinate treated suspensions (Figure 4D,E), suggesting neutrophils are not activated by succinate treatment. Also, whole blood supplied with succinate showed a higher level of platelet-PMN adherence (Figure 4G), in line with our observation of P-selectin surface expression and LTC_4 production in succinate-treated platelets and platelet PMN suspension.

Of note, when synthetic exogenous LTA_4 (10 µmol/L) was used as the substrate source, PRP incubated with succinate produced more LTC_4 than control (Figure 4F), suggesting succinate increases LTC4S activity in platelets. LTC4S is post-translationally regulated by phosphorylation that reduces enzyme activity.[23] Therefore, one may speculate that succinate can activate a protein phosphatase acting on LTC4S resulting in enhanced enzyme activity. Further studies will be required to understand the mechanism underlying the effect of succinate on LTC4S activation.

3.5 | Therapeutic implications of succinate-GPR91-eicosanoid signaling

Our data add GPR91 to at least three previously identified levels at which pharmacological intervention could reduce platelet activation and aggregation (COX-1, the platelet TXA_2 receptor, and 12-LOX). Low-dose ASA targets COX and is widely used for this purpose, while thromboxane receptor antagonists have been developed without making it to clinical settings. 12-LOX and 12-HETE have been implicated in platelet functions and thrombus formation[24] and recently, ML355, a potent and selective 12-LOX inhibitor, was suggested to impair platelet reactivity and clotting.[25] In fact, as ASA could only partially inhibit succinate potentiated platelet aggregation[12] (Figure 3B), the effectiveness of ML355 in

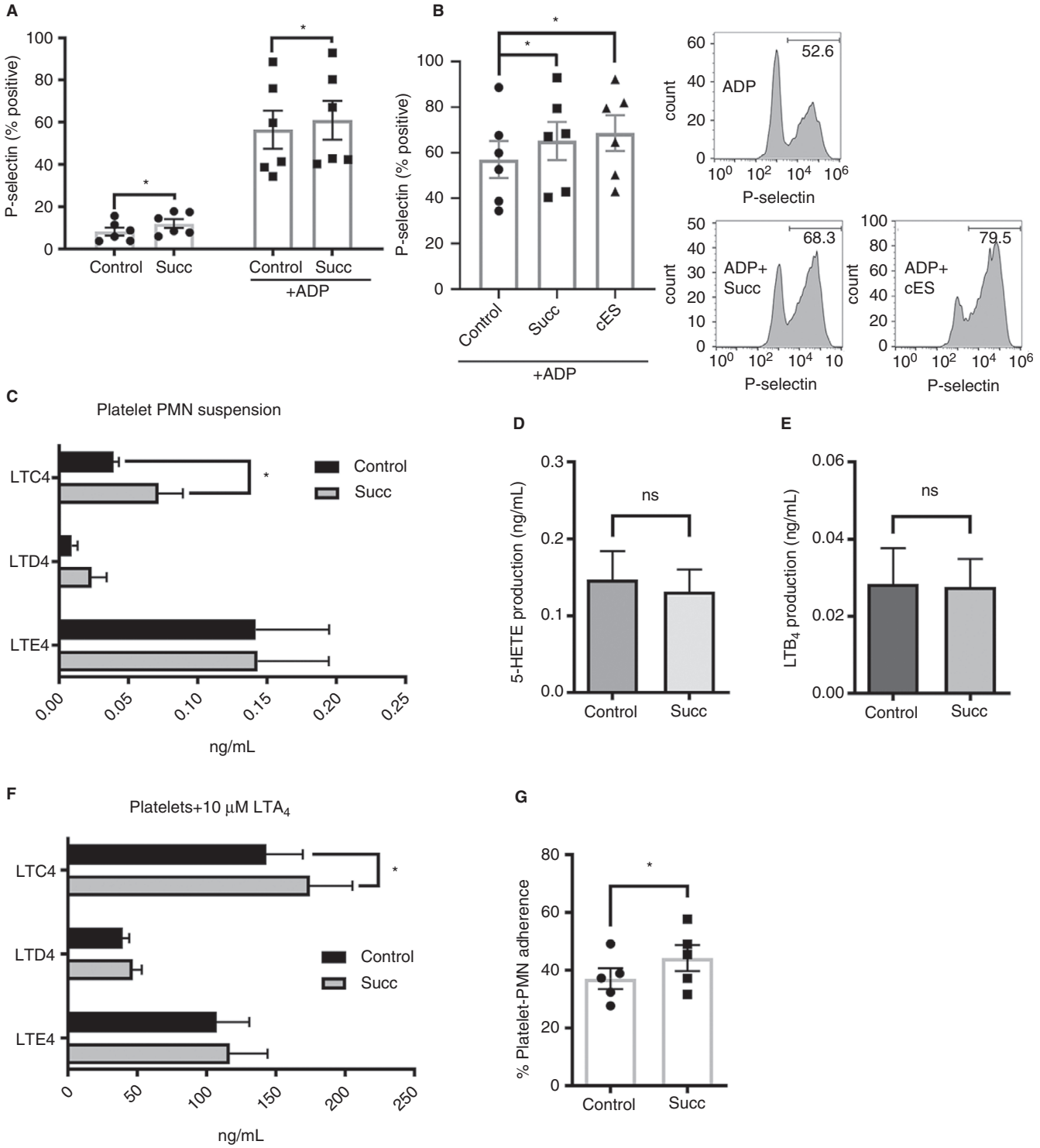

FIGURE 4 Succinate promotes transcellular biosynthesis of leukotriene C_4 (LTC_4). A-B, Whole blood was incubated with various compounds in HEPES buffer with fluorescence labeled P-selectin antibody at room temperature for 20 minutes, the cells were then fixed with 0.5% paraformaldehyde (PFA) and analyzed by fluorescence-activated cell sorting (FACS). C-E, Platelets and platelet-polymorphonuclear leukocytes (PMNs) were cocultured with 1 mmol/L succinate at 37°C for 30 minutes. The reaction was stopped by two volumes of methanol and lipid mediators were analyzed by liquid chromatography-mass spectrometry (LC-MS)/MS. F, 10 μmol/L LTA_4 with or without 1 mmol/L succinate was added to platelets and incubated at 37°C for 30 minutes. The reaction was stopped by two volumes of methanol and the lipid mediators were analyzed by LC-MS/MS. G, Whole blood was incubated with various compounds in HEPES buffer with fluorescence labeled CD45 and CD42a antibodies at room temperature for 20 minutes, the cells were then fixed with 0.5% PFA and analyzed by FACS. *$P < .05$

the current study supports targeting 12-LOX as a potential new antiplatelet therapy, especially in patients with ASA resistance or intolerance.

Succinate activates platelets with increased surface exposure of P-selectin, which promotes formation of platelet-neutrophil aggregates[6,26] and transcellular biosynthesis of LTC_4.[27] This cooperative action could be an important source of LTC_4 under certain circumstances as both cell types are fast-reacting components in inflammation, especially in aspirin-exacerbated respiratory disease.[28] In fact, this notion can be extended to atherosclerosis and associated cardiovascular diseases. For instance, the recruitment of neutrophils into atherosclerotic lesion is partially dependent on activated platelets, which further promotes early lesional development of atherosclerosis.[29] Another example is the vulnerable atherosclerotic plaque, which is prone to rupture and thrombus formation. Although the pathogenic mechanism(s) are not well understood it is believed to involve exposure of plaque lipids and immune cells, which in turn activates platelets leading to clotting, a scenario potentially involving transcellular biosynthesis of leukotrienes.

The autocrine and paracrine regulatory mechanism through which succinate acts as a ligand for GPR91 now introduces a novel possibility for interfering with platelet activation. Succinate is generated and excreted by many different cell types under metabolic stress including hypoxia.[30] Here we find that succinate promotes platelet activation and aggregation as well as transcellular biosynthesis of the pro-inflammatory LTC_4. Thus, LTC_4, the parent compound of the spasmogenic and immune regulatory cysteinyl-leukotrienes, could be formed when clotting platelets attach to inflammatory cells of, for example, the vulnerable plaque. It appears that GPR91 antagonists could be a novel means to block this process.

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CONFLICTS OF INTEREST

The manuscript has been read and approved for submission to JTH™ by all authors. The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

X. Tang contributed to the study design, performed the experiments, analyzed the data, and wrote the manuscript. S. Tan and N. Li contributed to the experimental design and interpretations. M. Trauelsen and T.W. Schwartz provided expertise in GPR91 pharmacology. D. Fuchs and C.E. Wheelock performed lipidomics. J.Z. Haeggström designed the study, analyzed the data, and wrote the manuscript.

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